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Culture-Amplified Detection of Dengue Virus From Serum in an Outbreak of Dengue Fever

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An outbreak of dengue type 2 occurred in North Queensland, Australia, between December 1996 and April 1997. Culture of serum in the *Aedes albopictus* C6/36 cell line with detection using immunofluorescent staining was compared with a culture-amplified detection system using an immunoperoxidase staining method in a microtiter plate format. A total of 374 serum specimens from individuals during the outbreak were tested. Ninety-five specimens were positive using immunofluorescence and ninety-two were positive using the immunoperoxidase method (sensitivity 91.6%; specificity 98.2%). The immunoperoxidase method is quicker, easier to perform, and does not require the use of an immunofluorescent microscope. The method is more suited to the processing of large numbers of specimens in an outbreak and could be used in endemic areas with limited virological resources. ***J. Med. Virol. 57:212–215, 1999.***

KEY WORDS: dengue; culture amplification; C6/36; horseradish peroxidase

INTRODUCTION

Dengue fever is an arboviral disease with important public health implications for many tropical countries. There are four serotypes of dengue virus, designated types 1 to 4. The risk of the potentially fatal complication of dengue hemorrhagic fever (DHF) increases with infection with a different serotype from a previous infection [Sangkawibha et al., 1984]. With the increase in international travel and increasing redistribution of people away from rural into urban centers, the introduction of different serotypes into new areas has increased. Early diagnosis and intervention can help reduce the morbidity and mortality from DHF.

There have been epidemics of dengue fever in North Queensland, Australia, since the late 19th century [Hare, 1898]. During the last forty-five years, epidemics of dengue have occurred in this region with increasing frequency. These epidemics were due to dengue 3 in 1953–1955 [Doherty et al., 1967]; dengue 1 in 1981–1982 [Kay et al., 1984]; dengue 1 in 1990–1991; dengue 2 in 1992–1993 [Streatfield et al., 1993]; dengue 2 in 1996–1997 [Hanna et al., 1998]; and most recently dengue 3, which began late in 1997 and is still ongoing as of May 1998. The outbreak in 1996–1997 affected the Torres Strait group of islands in North Queensland and was caused by dengue type 2 virus. More than two hundred people were affected in this outbreak. Approximately half were confirmed by culture and the remaining half by serology. This phenomenon is well recognized and reflects the timing of blood collection. Blood samples taken early in the course are more likely to contain virus and lack antibody, while the converse is true for later samples. This highlights the importance of both serology and culture for the diagnosis of dengue fever [Gubler et al., 1988].

There have been a number of viral culture methods described for the isolation of dengue viruses and include the use of live mosquitoes, mammalian cell lines, and cell lines derived from mosquitoes. The use of mosquito cell lines may be slightly less sensitive than live mosquito inoculation but have gained wide acceptance due to their convenience [Gubler et al., 1988]. The C6/36 mosquito cell line, derived from *Aedes albopictus,* sustains the growth of a number of arboviruses but does not generally develop visible cytopathic effects [Igarashi, 1978]. Detection of infected cells has been performed using immunofluorescent staining with either polyclonal mouse antibodies [Gubler et al., 1984] or a panel of monoclonal antibodies developed at the Walter Reed Army Institute of Research in the United States [Henchal et al., 1982]. Immunofluorescent staining has traditionally been performed after 7 to 10 days of incubation. In order to shorten the detection time and make the system more amenable to large numbers of specimens, a culture-amplified detection

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system using the same cell line in microtiter plates was developed. The incubation time was shortened to five days and an immunoperoxidase detection system was used. This method was compared with culture in separate flasks and immunofluorescent staining after 7 days.

MATERIALS AND METHODS

Population Sampled

Sera was obtained from 374 residents of the Torres Strait islands in North Queensland during an epidemic of dengue 2 in late 1996 and early 1997. The samples were submitted for diagnostic evaluation of dengue fever. The date of onset of illness and symptoms were, in most cases, not recorded by the requesting doctor. The group included both adults and children.

Culture-Amplified Detection Using Immunofluorescent Staining (IF)

One hundred microliters of serum was inoculated onto confluent monolayers of C6/36 (*A. albopictus*) cells in 25-cm^2 tissue culture flasks (Sarstedt, Australia) containing 5 ml of RPMI 1640 media (James Cook University, Tropical Biotechnology Pty Ltd, Australia) supplemented with 5% fetal bovine serum (FBS) (CSL Biosciences, Australia) and incubated at 28°C. At day 7 postinoculation, the culture fluid from each flask was decanted and the cell layer was washed with phosphate buffered saline (PBS) (Sigma-Aldrich, Australia) three times, leaving the last wash in the flask. Using a cotton swab, the cells were scraped from the surface of the flasks and resuspended in the PBS. The cell suspensions were decanted and centrifuged for 10 min at 2,500 rpm. The supernatant fluids were removed and the cell pellets resuspended in 5–10 ml of fresh PBS. The cell suspensions were dotted onto 15-well fluorescent antibody slides (ICN Biomedicals, Costa Mesa, CA). Slides were allowed to air dry, then were fixed in cold acetone for 10 min.

Antibodies were obtained from the Department of Molecular Sciences, James Cook University, Australia, and the James Cook University, Tropical Biotechnology Pty Ltd, Australia. A Flavivirus group antibody (4G2), a dengue virus group antibody (2H2), and four dengue type-specific antibodies 15F3 (DEN-1), 3H5 (DEN-2), 5D4 (DEN-3), and 1H10 (DEN-4) were used. All antibodies used were in the form of hybridoma fluid diluted 1:10, except for 2H2 (dengue virus group antibody) hyperimmune mouse ascitic fluid, which was diluted 1:1,000. The diluent was TEN-Tween (0.05-M Tris, 1-mM EDTA, 0.15 M NaCl, 0.05% v/v Tween 20) with 0.5% bovine serum albumin (James Cook University, Tropical Biotechnology Pty Ltd).

To the fixed cells were added 7 μl of each of the diluted antibodies. Following 30 min of incubation at 35°C, the slides were washed in PBS for 10 min and were then allowed to air dry. Fluorescein-conjugated goat antimouse antibodies (Cappell-Organon Teknika, Westchester, PA) were diluted 1:640 in PBS and Evans blue counterstain (FITC conjugate diluted in equal volumes of PBS and 0.02% Evan's Blue). Seven μl of this solution was added to each well. Slides were incubated for 30 min at 35°C, washed in PBS for 10 min, and again allowed to air dry. Slides were examined with fluorescence microscopy (Zeiss Axioskop, Carl Zeiss, W. Germany) using 400× magnification. Cells were examined for cytoplasmic fluorescence and virus identification was determined according to reactivity with specific monoclonal antibodies.

Culture-Amplified Detection Using a Horseradish Peroxidase (HRPO) Enzyme Immunoassay

Following the addition of serum to the culture flasks used in the immunofluorescent method, the flasks were tilted to mix the contents. Six 100-μl aliquots of this diluted serum were removed from the flasks and inoculated into wells of a 96-well tissue culture tray (Sarstedt), which contained confluent C6/36 cell monolayers and 100 μl of RPMI 1640 media supplemented with 5% FBS. After 5 days of incubation at 28°C, the culture fluid was removed from the wells and the cells were fixed for 15 min at room temperature with 200 μl of 1% paraformaldehyde, 0.1% Nonidet P40 in PBS per well. Following fixation, the plates were washed three times in TEN-Tween and drained. Fifty microliters of the same diluted monoclonal antibodies used for the immunofluorescent detection were added to the six wells of the microtiter tray and left for 1 hr at room temperature. After washing the plates three times in TEN-Tween, 50 μl of goat antimouse antibodies conjugated with horseradish peroxidase (Dako, Australia) diluted 1:1,000 in TEN-Tween with 0.5% bovine serum albumin were added. After 1 hr at room temperature the plates were again washed three times in TEN-Tween and drained. Cells were stained using 100 μl of 1-mM ethyl carbazole in 50-mM acetate buffer pH 5 plus 0.015% H_2O_2. Color development was allowed to proceed for 30 min and the plates were examined with an inverted light microscope. Wells that contained cells that developed a red-brown color were recorded as positive, whereas cells that remained colorless were negative. The use of both group and type-specific antibodies in one tray enabled detection and typing of the dengue virus.

RESULTS

Of the 374 serum specimens tested, 87 were positive for dengue type 2 virus using both the immunofluorescent and immunoperoxidase methods. There were eight additional specimens that were positive only by the immunofluorescent method and five specimens that were positive by the immunoperoxidase method only. These results are shown in Table I. The sensitivity and specificity of the immunoperoxidase method compared with the immunofluorescent method was 91.6% and 98.2%, respectively. A positive result using the immunoperoxidase method was generally easy to interpret. In most cases, the whole cell monolayer was infected and infected wells could be observed with the naked eye. When the monolayer was less heavily in-

TABLE I. Results of Dengue Testing[a]

Detection method	Culture-amplified detection with IF-positive	Culture-amplified detection with IF-negative	Total
Culture-amplified detection with HRPO-positive	87	5	92
Culture-amplified detection with HRPO-negative	8	274	282
Total	95	279	374

[a]Sensitivity, 91.6%; specificity, 98.2%; positive predictive value (PPV), 94.6%; negative predictive value (NPV), 97%.

fected there were usually several plaques of stained cells, each plaque comprising at least a dozen cells. Very rarely, individual infected cells were observed, but were usually accompanied by plaques elsewhere in the monolayer. Interpretation was generally easy and did not appear to be affected by low-level background staining. When infection occurred, there were always infected cells in the wells where three of the monoclonals, 4G2, 2H2, and 3H5, were used. It was therefore possible to screen only the wells that had used 4G2 as the primary antibody. Up to 16 serum samples were tested using one 96-well tissue culture plate and one plate could be evaluated in less than 10 min. In 87 of the 92 specimens positive using the immunoperoxidase method (94.6%), the immunofluorescent test was also positive, which meant that in most cases the viral isolate was present in the cell culture media retrieved from the cell culture flask.

DICUSSION

Dengue fever is an arboviral disease of considerable regional importance. Public health measures need to be implemented early to limit the spread of this infection. Early diagnosis is therefore of considerable importance and relies on a combination of clinical suspicion in the appropriate epidemiological setting and laboratory confirmation. Serology alone is only of value more than about 5 days after the onset of the illness. Most patients, however, present earlier in the course of their illness and may not return for venesection after seroconversion. Viral culture, therefore, plays an important part in the early diagnosis of this condition as well as providing information concerning the serotype of the infection and are a source of viral isolates, which may be used in molecular epidemiology studies. More recently, a number of studies have demonstrated the utility of reverse transcription–polymerase chain reaction (RT-PCR) [Morita et al., 1991; Lanciotti et al., 1992; Chungue et al., 1993; Seah et al., 1995]. RT-PCR, however, remains relatively labor-intensive and expensive compared with viral culture and does not yield a viral isolate.

Culture-amplified detection systems using a horseradish peroxidase-based enzyme immunoassay have not previously been described in the diagnosis of dengue, although immunohistochemical stains have been used to detect dengue-infected cells in formalin-fixed tissue specimens [Hall et al., 1991]. This study demonstrates that the immunoperoxidase culture-amplified detection system is of comparable sensitivity to the traditional flask-based culture with immunofluorescent detection. Advantages of such a system include earlier confirmation of culture-positive sera and the convenience of the method. Relatively little equipment is required for the method; in particular, there is no requirement for a fluorescence microscope. The microtiter plate format is suitable for the cost-efficient processing of large specimen numbers. One criticism of immunoperoxidase staining is the presence of background stain possibly due to endogenous peroxidase. In practice, this is seldom a problem and, as shown in this study, differences in the intensity and pattern of staining are easily discernible.

The sensitivity of the method might have been higher had the incubation time been extended. However, one of the major advantages of this method was the shorter incubation time. The effective inoculum of serum into each well of 96-well tissue culture was about 2 microliters compared with the 100 microliters used to infect the 25 cm^2 flask. It was somewhat surprising that the discrepancy in the inoculum size should have such a modest effect on sensitivity. Less easy to explain were the five samples found to be positive using the immunoperoxidase method that were negative on immunofluorescence.

The immunoperoxidase method is an economical and sensitive method for the detection of dengue viremia and could be considered for laboratories that have not previously attempted viral culture. Such laboratories are common in areas where dengue fever is endemic and/or epidemic. Rapid detection of dengue viremia in these areas is an essential accompaniment to the public health response to dengue fever.

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